THE BACTERIOLOGICAL EXAMINATION OF A PLAGUE RAT, WITH NOTES ON THE CAPSULAR SUBSTANCE FORMED ON NUTRIENT AGAR BY SOME BACTERIA.*†

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ON March 15, 1905, a rat found dead in the house of Dr. Lugay, 106 Calle Principe, was sent to the Laboratory by Dr. W. P. Read, Station A, San Nicholas, Manila. I had never felt satisfied that plague occurred in rats in Manila, so the cultures isolated were subjected to a careful study.

The routine examination of a large number of rats becomes so onerous that most laboratories adopt some rough and ready methods of diagnosis. In Manila the diagnoses were based on microscopic examinations. In Japan and China (Hong Kong) similar methods have been relied upon. In India less questionable methods are in vogue, the formation of involution forms on salt agar being relied upon in Central India (Hankin), while in the Plague Research Laboratories at Parel, Bombay, the formation of stalactites in flasks of broth is the criterion.

While the use of one, or better both, of the last two methods is all that can be expected when a large number of rats must be examined, it would certainly seem advisable to supplement this work by a few more carefully conducted bacteriologic examinations, especially when plague first appears in a new locus, or when only a few rats are found dying of a plaguelike disease.‡

An acquaintance with the work of Dunbar and Kister,[1] and especially with that of Neumann,[2] on the occurrence of plaguelike microorganisms in rats will emphasize the point I wish to make. One grows tired of reading of the occurrence of plague in pigs,

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‡ In Manila (1903) out of 1,623 rats examined, one-sixteenth of 1 per cent were found infected with plaguelike bacteria.

[1] *Centralbl. f. Bakt.*, 1904, 36, p. 127.

[2] *Zeit. f. Hyg.*, 1903, 45, p. 450.

dogs, jackals, snakes, etc., without the presentation of sufficient evidence in support of the statements.

BACTERIOLOGICAL EXAMINATION.

The rat was an adult male *Mus rattus.** No fleas or lice were found, but it was covered by a large number of red ants; slight edema in subcutaneous tissues of chest; superficial glands not enlarged; peritoneal cavity normal; spleen slightly enlarged; liver congested, and on its surface numerous pin point sized, whitish areas of necrosis; lungs slightly congested; a little fluid in peritoneal and thoracic cavities. Smears from the liver and spleen, stained with Wright's modification of the Romanowski,[1] showed numerous bi-polar staining rods. (See Fig. 1.) All bacteria in preparations from the spleen and liver gave up the stain in Gram's method. Successive stroke cultures were made from the spleen and liver on +1 agar† slants and kept at 36–37°. In 24 hours quite a number of colonies had appeared. These reached a diameter of 2–4 mm. in 48 hours and were not viscous when touched with a platinum needle. As cultures from these produced indol in broth, coagulated milk, and fermented dextrose, levulose, lactose, and saccharose with gas production, they were discarded as agonal or post-mortem invaders of the colon group. Besides these there developed on the slants from the spleen a few colonies of a diplococcus which closely resembled the variety of

*It is generally accepted that the rat is the original host of B. pestis; and however interesting it may be to know the percentage of rats infected during an epidemic in any locality, the importance of knowing what *kind* of rats are chiefly to blame is becoming more apparent every day. Bruce Skinner has presented evidence which seems to point to the complete or partial immunity of the Norway rat (*M. decumanus*) to naturally acquired plague, and has suggested that, through its successful antagonism to the long-tailed rat (*M. rattus*), it has played an important part in preventing the spread of plague in Europe and elsewhere. It would be interesting to know if such is the case in America. According to D. S. Jordan, *M. decumanus* was "introduced into America about 1775, and is now the commonest species, having nearly exterminated the next" (*M. rattus*). Bacteriologic examinations by Liston show that *M. decumanus* also carries plague, but, owing to its domestic habits, *M. rattus* is a greater menace to man. See W. G. LISTON, "Plague Rats and Fleas," *Ind. Med. Gaz.*, 1905, 40, p. 43, and "The Rats of India," *ibid.*, 1905, 40, p. 130.

[1] *Jour. Med. Res.*, 1902, 7, p. 138.

†Wilson (*Jour. Med. Res.*, 1901, 6, p. 53) showed that 1 per cent acid to phenolphthalein is the optimum reaction for B. pestis, the rapidity and amount of growth diminishing progressively with the increase of acid or alkali. My media were prepared according to the recommendation of the American committee, except that the reaction to phenolphthalein was adjusted by the addition of sodium hydroxide alone. (See *Bull. 19*, Bu. of Gov't Labs., Manila, P. I., 1904.)

Micrococcus catarrhalis, described by Lyon and myself from a case of chronic dermatitis.[1]

In 48 hours, however, numerous discrete, pin-point sized, transparent colonies could be seen between the larger ones. Under the hand lens these appeared round, raised, and perfectly transparent, exactly like a non-saprophytic growth of B. bovisepticus. Transplants on +1 agar slants were made from these minute colonies and marked Transplant (1) Spleen (d) and Transplant (1) Liver (d), respectively.

Wright's modification of the Romanowski had on previous occasions yielded such beautiful results in demonstrating the bi-polar staining of B. pestis in preparations from animal tissues that I thought it might yield similar results in preparations from cultures. Very thin films (in distilled water) were prepared from the fine colonies described above as Spleen (d) and Liver (d), dried in the air, and stained in the manner usually employed in staining a film of blood. The result was very surprising; the rods took up the methylene blue uniformly, and many of them were surrounded by a well-defined oval, capsular substance which took the eosin stain. (See Fig. 2.)

The demonstration of what appeared to be a capsular substance, taken along with the delay in the appearance of the primary growth from the tissues, brought to my mind the theory concerning the production of defensive capsules by bacteria presented by Dr. Theobald Smith at the St. Louis Congress of Arts and Sciences.[2] So an effort was made to keep a strict account of each transplant of cultures Liver (d) and Spleen (d), with the idea of noting whether the subcultures changed in any particular manner during cultivation, or if the capsular substance increased or decreased in amount, or changed in any way in its microchemical reactions.

The subcultures were made on freshly prepared +1 agar slants (made up in several different lots and varying slightly in reaction and color and perhaps in composition) and kept at 36–37° until growth had become quite evident to the naked eye (24 to 48 hours), when stained preparations were made. The

[1] *American Medicine*, 1903, 6, p. 401. [2] *American Medicine*, 1904, 8, p. 711.

original subculture was then in each instance kept on a shelf at 18–28° and used for inoculating another subculture or an animal or discarded, as the case might be.

Briefly, the history of Liver (d) may be given as follows:

Liver (d), inoculated from tissue on March 15, capsules with Wright's Romanowski.													
Transplant	(1)	"	"	"	"	"	"	"	17,	"	"	"	"
"	(2)	"	"	"	"	"	"	"	19,	"	"	"	"
"	(3)	"	"	"	"	"	"	"	22,	"	"	"	"
"	(4)	"	"	"	"	"	"	"	26,	"	"	"	"
"	(5)	"	"	"	"	"	"	"	28,	"	"	"	"

As already stated, the tubes inoculated from the rat's liver showed none of the minute transparent colonies after 24 hours' incubation at 36–37°, but a plainly visible growth was present in 48 hours. In the case of Transplant (1), no visible growth was present after 24 hours at 36–37°, with the possible exception of two or three scattered colonies. The water of condensation was allowed to flow over the surface of the agar slant, and in 48 hours numerous transparent, pin-point sized colonies were present. In the case of Transplants (2), (3), (4), and (5), a very delicate confluent growth appeared after 24 hours' incubation at 36–37°; but it must be noted that the successive subcultures were not made at exactly equal intervals of time.

No peculiar changes in the capsular substance were noted. Owing to my departure I was unable to follow this system through any extended period of time. However, a staining from a culture of B. pestis over two years old (described below as No. 15) showed that the capsular substance was not lost with an increase in the saprophytic powers of the culture. (See Fig. 3.)

The number of bacteria showing this capsular substance seemed to vary somewhat in different preparations. As a rule the organisms lying in the better-spread portions of a preparation showed it most plainly, and though sometimes the capsular substance stained evenly with eosin, at other times it would be merely outlined by a reddish-purple halo. Enough work was not done to prove that this was not due to slight variations in technique.

Culture Spleen (d) in the course of a few transplants grew more luxuriantly than is usual with recently isolated cultures of B. pestis, and losing its

viscosity it took on a granular consistency. A very small amount of the first transplant killed guinea-pig 1132 (numerous bi-polar staining rods). As much as 2 mg. of Transplant (7) failed to kill guinea-pigs. As the culture produced indol and fermented sugars it was discarded as overgrown by B. coli.

BIOCHEMICAL CHARACTER OF CULTURE LIVER (d).

Transplant (4) was inoculated into litmus milk, +1 broth, and Dunham's peptone solution; both of the latter contained a small amount of nitrite. The litmus milk was unchanged, and no indol could be demonstrated after five days at 36–37°.

When grown in +1 broth containing 1 per cent of dextrose, levulose, lactose, saccharose and starch (Kahlbaum's), in the fermentation tube, in each instance, at 36–37°, growth appeared only in the bulb and open neck, i. e., aerobically.

In order to make the specificity of this culture more certain, it was compared in several of its more important biochemical characteristics with the following cultures of B. pestis derived from human sources:

Culture 15.—Isolated by myself from spleen, Autopsy 532 March, 1903. Since then it has been transplanted on +1 agar slants eight or nine times and kept in the ice chest. Grows slowly in the ice chest. Growth, at first delicate, is now quite luxuriant and viscous. Pathogenic for guinea-pigs by scarification at time of isolation.

The marked polymorphism and variation in size shown by the same or different cultures of B. pestis is well illustrated on comparing Fig. 2 with Figs. 1 and 3.

Culture 26.—Is identical with Culture 15, excepting that the virulence of this particular strain was kept up by passage through guinea-pigs until about three months ago. Since then it has been kept in the ice chest. Killed guinea-pigs regularly, by scarification, in four or five days. It has been used by Dr. E. H. Ruediger in immunizing Horse 12 (*vide infra*). Growth is quite luxuriant. Preparations from +1 agar slants show capsules with Wright's Romanowski.

Culture H.—Isolated from a human plague case about February 1905 by Dr. Herzog. Its growth is only moderately

luxuriant. Preparations from +1 agar slants show capsules with Wright's Romanowski.

1. *With regard to the production of involution forms on fresh +1 agar slants containing 2 or 3 per cent of Kahlbaum's chemically pure sodium chloride (proved free from nitrates or nitrites).*—All of these cultures produced an extremely delicate growth after 24 hours at 36–37°. Stained preparations showed large sinuous and clubbed forms which were very much alike throughout the series of cultures. (See Fig. 4.) The use of 2 or 3 per cent of the sodium chloride made no appreciable difference in the character of the growths or polymorphism shown by any particular culture.

2. *With regard to the formation of stalactites in oiled broth.*—A thin layer of cocoanut oil was placed in +1 broth, the tubes sterilized at 120°, inoculated, and kept in a perfectly quiet place at 18–28°. Culture 15 produced stalactites in three days; 26 in four days; and H in about a week. Liver (d) Transplant (2) scarcely grew at all in the broth and produced no stalactites during 12 days' observation.

A number of workers have recorded an inconsistency in the production of stalactites shown by cultures of B. pestis. Theoretically one should be able, by artificial selection, to train such a culture of B. pestis to produce stalactites, just as it is possible to train cultures of cholera, diphtheria, etc., to produce pellicles in broth cultures.[1] According to Lieut.-Col. Bannerman, Director of the Plague Research Laboratories, Bombay, the stalactite growth never fails to appear when flasks of broth containing a drop or two of "ghee" (clarified butter) or cocoanut oil are "seeded" with material taken directly from fresh plague buboes.

3. *With regard to their agglutination with antiplague serum.*—The serum of Horse 12, immunized against culture 26, was kindly furnished by Dr. E. H. Ruediger of the Serum Institute. The cultures, grown for three days on +1 agar slants at 36–37°, were suspended in 0.85 per cent sodium chloride solution, and the suspensions then brought to approximately the same density by further dilution with the salt solution. The serum was diluted

[1] See *Bull. 19*, Bu. Govt. Labs., Manila, P. I., 1904.

with the same salt solution, and one c.c. of each dilution of the serum added to one c.c. of the culture suspensions in small test tubes and kept at 36–37°. The controls made up with salt solution alone were kept under the same conditions. The results are shown in the following table, where + indicates complete precipitation, ± precipitation with a clouded fluid above, and — no precipitation.

Dilution of Serum.....	1/10		1/100		1/200		1/400		1/1000	
Hours at 36-37°.....	16	24	16	24	16	24	16	24	16	24
Culture 26	±	+	—	±	—	—	—	—	—	—
Culture 15	±	+	—	±	—	—	—	—	—	—
Culture H	±	+	—	±	—	—	—	—	—	—
Culture Liver (d).....	—	±	—	—	—	—	—	—	—	—
Culture Spleen (d)*....	+	+	±	+	±	±	±	±	—	—

Controls all negative throughout.

In another test with the same series of cultures, Culture Liver (d) was completely precipitated in 24 hours at 1:10 and 1:20 dilutions.

INOCULATION EXPERIMENTS.

The pathogenicity of the cultures isolated was tested upon a number of guinea-pigs and wild rats, but I will insert only a few protocols to demonstrate the pathogenicity of Culture Liver (d).

1. *Guinea-pig 1129.*—Abdomen washed, shaved, and scarified with a sterile needle. Rubbed in a small amount of the growth of very minute colonies on the original +1 agar culture from liver of rat. (Colonies 48 hours old.) On fourth day slight inflammatory reaction about site of scarification; no enlargement of inguinal glands. On seventh day local reaction increased, raised, reddened; no palpable enlargement of inguinal glands. On ninth day very sick and listless, eyelids gummed together. Chloroformed.

Post mortem.—Oval thickened area of infiltration which is in the right lower quadrant of the abdomen; right inguinal glands 6–8 mm. in diameter and imbedded in an area of gelatinous edema; on section glands show pink and yellowish caseous contents; spleen five or six times normal size, soft, and contains many yellowish nodules 2–3 mm. in diameter; liver congested and contains many nodules as in spleen; kidneys congested; lungs show irregular areas of consolidation which show a reddish-gray surface when cut; no hemorrhages.

*Since this Culture Spleen (d), as stated above, was overgrown by a member of the colon group, this agglutination with antiplague serum might seem remarkable were it not for what we know concerning the absorption of agglutinins for various strains of B. coli from the intestinal tract during the course of certain fevers, e. g., typhoid, or during active immunization with various cultures (See Park and Collins, *Jour. Med. Res.*, 1904, 12, p. 491).

Smears from the skin wound, inguinal glands and lungs showed numerous polar staining rods (many of which were involution forms). No bacteria were seen in the preparations from the spleen and liver (perhaps because they were shut up in the caseous foci which were not broken up in obtaining material for the smears?).

Cultures on +1 agar slants kept at 36–37°. Those inoculated from the local skin wound and liver showed numerous very minute transparent colonies after 24 hours. A loop of blood from the heart gave no growth. A few colonies appeared in 48 hours on slants inoculated from the spleen. Bacteria from these colonies gave the capsule stain with Wright's Romanowski.

2. *Guinea-pig 1131.*—A very small amount of the same culture used in inoculating Guinea-pig 1129 was injected into the peritoneal cavity of this animal. Chloroformed in a dying condition on the seventh day.

Post mortem.—Where the needle entered in the lower left quadrant of the abdomen is a yellowish, caseous, subcutaneous. and intramuscular nodule about 4 mm. in diameter; inguinal glands on left side enlarged, congested, surrounded by a gelatinous edema; right inguinal glands somewhat enlarged; axillary glands apparently not affected; liver congested and shows a few pinhead sized caseous areas; spleen enlarged to 2½ in. by 1 in. by ¼ in., soft and filled with irregular, yellowish-white, caseous areas; caseous nodules along sternum; lungs congested; few pea sized, caseous nodules in abdominal cavity (necrotic glands?); colon greatly thickened, mucosa covered by bloody mucus and contents mucoid with streaks of blood. No hemorrhages.

Smears.—Numerous polar staining rods found in caseous nodules, liver, and spleen. Fresh preparation from colon shows a large number of amebæ-like bodies, non-motile but containing red blood cells and fragments of red blood corpuscles, a few trichomonas, and an organism which looks like the *Megastoma entericum.*

Cultures.—The organism was recovered on agar slants from the heart's blood, spleen, and liver. Time when growth on agar slants appeared not noticed. (N. B.—Tissues and cultures from the guinea-pig's colon were turned over to Dr. Musgrave and Dr. Woolley, who will report on the case if it should prove to be one of spontaneous amebic dysentery in a guinea-pig.)

3. *Rat. 3.*—Large adult male (*Mus decumanus?*). Received intraperitoneal injection of a small amount of Liver (d) Transplant (2) suspended in 0.8 per cent salt solution. Dead in less than 48 hours.

Post mortem.—No fleas or lice found; congestion and swelling of the spleen; congestion of the liver; double, clear, serous hydrothorax; atelectasis of the lungs; no hemorrhages.

Smears from liver and spleen show large numbers of polar staining rods. Rods comparatively fewer in serous fluid, where they occur singly and in chains of 6–8 individuals.

Cultures.—The minute colonies from spleen show capsulated rods when stained with Wright's Romanowski. Time when growth appeared on agar slants not noticed.

4. *Rat 4.*—Adult female (*Mus decumanus?*). Fed on some bread soaked in well-clouded emulsion from which Rat 3 was injected. It remained well during two weeks' observation.

GENERAL CONSIDERATIONS.

It seems to be entirely too soon to speculate on the significance of the production of this capsular substance on nutrient agar. Several questions present themselves: Is it really an integral part of the bacterial cell? Will all bacteria show it, or is it only produced under certain conditions by particular groups of pathogenic bacteria? Is it produced in fluid, as well as on solid, media? If it cannot be demonstrated in animal tissues by the same technical methods, does this fact partly answer the question: "Do the parasites act differently when posing for us in the culture tube from the way they behave in the animal body?"

I do not believe that this capsular substance could be agar precipitated about the bacterial cell, for with this thought in mind I was very careful to touch only just the surface of the growth with the platinum needle; and then the size and shape of the capsule is too uniform to make one think of artifacts.

Through the kindness of Dr. Paul G. Woolley I was able to test this method on a culture of B. bovisepticus recently (48 hours) isolated from a tumor-like growth in the lung of a Chinese bull. This culture also showed a delay in the appearance of the primary growth on +1 agar. A very clearly defined, capsular substance surrounds the rods, as shown in Fig. 5. It is interesting at least that, after fixation with methyl alcohol, one part of the organism should take the nuclear, and the other the protoplasmic, stain. No attempt was made to stain the capsular substance on bacteria in fluid cultures. Even prolonged immersion failed to stain capsules on B. pestis in smears from inoculated animals, though, as is well known, they often appear surrounded by an unstained halo.*

The particular portion of Dr. Theobald Smith's article which suggested the above work I may quote as follows:

*Capt. S. R. Douglas, R.A.M.C., of St. Mary's Hospital, London, informs me that capsules may be stained with Leishmann's dye, on B. pestis, in smears from the buboes of rats inoculated by the cutaneous method. Here some of the rods appear to be enveloped in a capsule stained pale blue, the bacilli themselves being almost black. "This capsule-like appearance was far more frequently found in wipes made from the buboes of rats than in those made from the spleens, but it was occasionally found in spleen wipes. I was never able to satisfy myself whether this appearance was found more frequently in partially immunized rats or in normal control rats, as the staining of these capsules was uncertain."

I am inclined to believe, however, that among the problems of the future will be the elucidation of still another mechanism for the protection and escape of the microorganism. It is highly probable that in a certain number of species of bacteria after the active vegetative stage a latent stage follows, during which the parasite which has escaped destruction provides itself with some protective envelope which also aids it in its passage to a new host. This envelope, which may be some species of substance not recognizable with the microscope, or which may be represented by the capsules in some groups, may be a defensive body of the parasite stimulated to overproduction by the antibodies of the host. This body also interferes with the metabolism of the microbe, and thus acts in the double capacity of stopping the disease and protecting the microbe at the same time. This hypothesis suggested itself to me while endeavoring to account for the peculiar behavior of tubercle bacilli under cultivation.

It is well known that tubercle bacilli from the diseased tissues of cattle grow very slowly and then only upon certain culture media such as blood-serum. After several years of continuous cultivation they multiply vigorously in glycerin bouillon, and can hardly be distinguished in appearance from those human varieties of the bacillus which grow richly from the first or second transfer. There seemed to be no justification to assume that the bacillus had completely changed its metabolism under artificial cultivation. The more rational hypothesis seemed to be the one which assumed the existence of some protective substance only slightly acted upon by the serum, not at all in glycerin bouillon, and therefore a hindrance to multiplication. After repeated transfers, this protective substance was slowly lost, either through a selection of bacilli, or absence of stimulation on the part of the host, or both causes combined, and the growth became as luxuriant as with the more saprophytic human varieties. It is obvious that each group or species of bacteria would have its own special protective device depending upon original capacities of the species which would be gradually developed in power and efficiency with the perfection of parasitic relations.

The formation of protective or defensive coverings, the strengthening or modification of the cell wall, or the secretion of defensive fluids, would account for certain phenomena, which are familiar to bacteriologists, much better than the current theory which bases parasitism exclusively upon toxin production, active or passive.

In cultures we should expect a loss of power to form protective substances because the antibodies are absent. Hence the universal tendency toward the reduction and final loss of virulence with apparently the metabolic and vegetative activities unchanged, and the frequently observed regaining of virulence by passages through a series of animals.

In my own experience the delay in the appearance of the primary growth of certain cultures (B. mallei, B. pestis, and certain members of the Hemorrhagic Septicemia Group of Hueppe) occurs only when bacteria are transferred either directly, or

through an experimental animal, from a naturally acquired form of disease to such an entirely new physical and chemical environment as is furnished by an agar slant; in which case it may be assumed, according to this theory, that the microorganisms are descended from an infinite number of ancestors especially adapted for growth in animal tissues.

But if such an organism once thoroughly "adapted" for growth on nutrient agar is inoculated into an experimental animal and then recovered from the tissues of this animal, here it will be seen that the time element plays an important part; for if the animal dies before the microorganism loses its newly acquired modification, the culture inoculated upon agar slants usually appears within 24 hours.

It is interesting to note that Culture Liver (d), though but one "generation" removed from the tissues of *Mus rattus*, produced a chronic disease in guinea-pigs, as shown by the marked reaction on the part of the tissues of the inoculated animals, while in the case of Rat 3 an acute general infection resulted. Cultures derived from human sources show some variation in their pathogenicity for guinea-pigs, but as a rule recently isolated cultures of B. pestis, when rubbed into a scratch on the abdomen of guinea-pigs, produce septicemia and death in four or five days with little or no tissue reaction apart from the local reaction, lymphadenitis, and splenic tumor. Unfortunately I had not time to conduct a more comparable series of animal inoculations which might help to elucidate a question which seems to me to be of epidemiological interest, i. e.: Are strains of B. pestis which have been adapted for growth in the tissues of the rat for perhaps an infinite number of "generations" as infective for other animals, e. g., guinea-pigs, monkeys, and man, as strains derived from human sources?

DESCRIPTION OF PHOTOMICROGRAPHS.

(Taken by Chas. Martin, Photographer, Bureau of Government Laboratories.)

All the preparations were stained by Wright's modification of the Romanowski. The magnifications were determined by measuring the projected image of a 1/100 mm. stage micrometer. Figs. 1, 2, 4, and 5 are reduced $\frac{1}{6}$; Fig. 3, $\frac{3}{18}$.

FIG. 1.—Smear from the rat's liver, showing the bi-polar staining of B. pestis, × 720.

FIG. 2.—Liver (d), Transplant (3), 24 hours at 36–37° on +1 agar slant, × 720.

FIG. 3.—Plague 15, 24 hours at 36–37° on +1 agar slant, × 700.

FIG. 4.—Liver (d), Transplant (2), 24 hours at 36–37° on +1 agar slant containing 3 per cent of chemically pure sodium chloride, × 720. By comparison with Figs. 1 and 2 it will be seen what a relatively enormous size these involution forms may attain.

FIG. 5.—From a culture of B. bovisepticus, 48 hours on +1 agar slant at 36–37°, × 720.

PLATE 19.

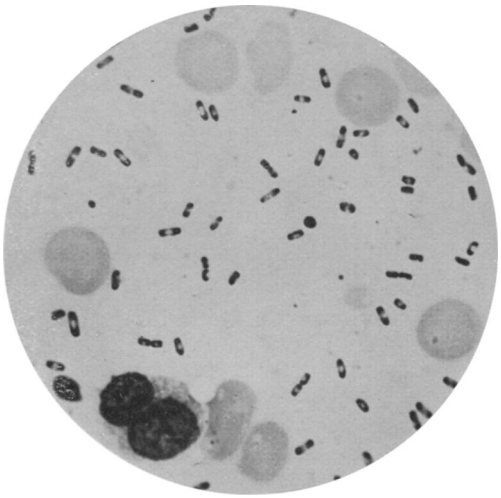

FIG. 1.

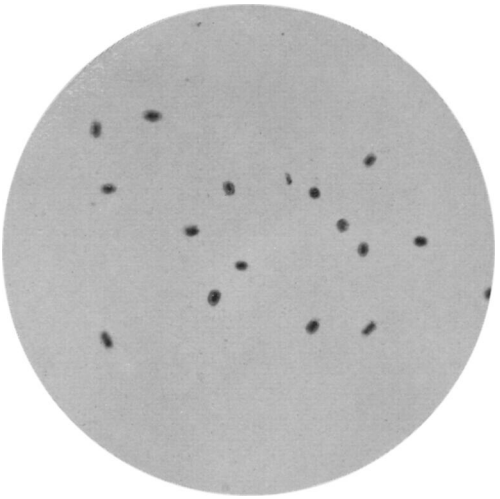

FIG. 2.

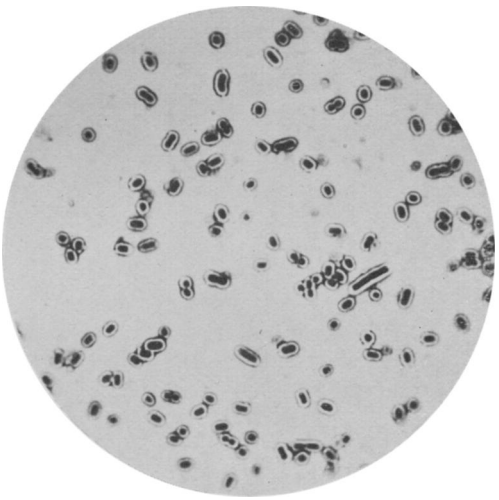

FIG. 3.

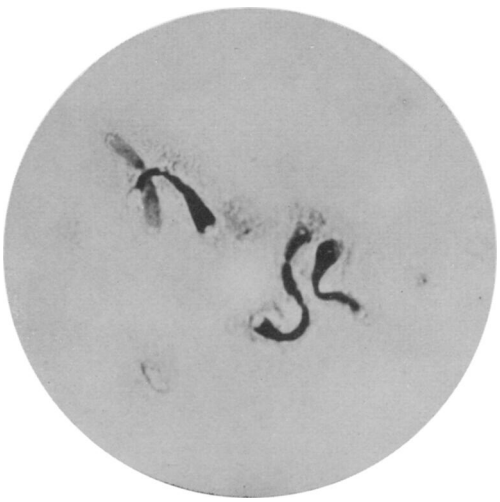

FIG. 4.

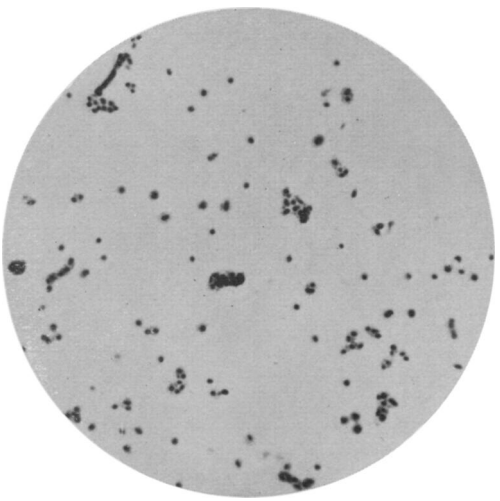

FIG. 5.